Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com

Glucose, amino acids and fatty acids directly regulate ghrelin and NUCB2/nesfatin-1 in the intestine and hepatopancreas of goldfish (*Carassius auratus*) *in vitro*

Juan Ignacio Bertucci [a], Ayelén Melisa Blanco [c], Luis Fabián Canosa [a, *], Suraj Unniappan [b, **]

[a] *Instituto de Investigaciones Biotecnológicas-Instituto Tecnológico Chascomús, Av. Intendente Marinos Km 8.2, CC 164 (7130) Chascomús, Prov. de Buenos Aires, Argentina*
[b] *Laboratory of Integrative Neuroendocrinology, Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, 52 Campus Drive, Saskatoon, Saskatchewan S7N 5B4, Canada*
[c] *Departamento de Fisiología (Fisiología Animal II), Facultad de Biología, Universidad Complutense de Madrid, Calle José Antonio Nováis 12, 28040 Madrid, Spain*

ARTICLE INFO

Article history:
Received 5 October 2016
Received in revised form 10 January 2017
Accepted 11 January 2017
Available online xxx

Keywords:
Fatty acids
Food intake
Ghrelin
Glucose
Macronutrients
Metabolism
NUCB2/nesfatin-1
Tryptophan

ABSTRACT

Ghrelin and nesfatin-1 are two peptidyl hormones primarily involved in food intake regulation. We previously reported that the amount of dietary carbohydrates, protein and lipids modulates the expression of these peptides in goldfish *in vivo*. In the present work, we aimed to characterize the effects of single nutrients on ghrelin and nesfatin-1 in the intestine and hepatopancreas. First, immunolocalization of ghrelin and NUCB2/nesfatin-1 in goldfish hepatopancreas cells was studied by immunohistochemistry. Second, the effects of 2 and 4 hour-long exposures of cultured intestine and hepatopancreas sections to glucose, L-tryptophan, oleic acid, linolenic acid (LNA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on ghrelin and nesfatin-1 gene and protein expression were studied. Co-localization of ghrelin and NUCB2/nesfatin-1 in the cytoplasm of goldfish hepatocytes was found. Exposure to glucose led to an upregulation of preproghrelin and a downregulation of *nucb2/nesfatin-1* in the intestine. L-Tryptophan mainly decreased the expression of both peptides in intestine and hepatopancreas. All fatty acids in general downregulated NUCB2/nesfatin-1 in the intestine, but only the longer and highly unsaturated fatty acids inhibit *preproghrelin*. EPA exposure led to a decrease in *preproghrelin*, and an increase in *nucb2/nesfatin-1* expression in hepatopancreas after 2 h. These results show that macronutrients exert a dose- and time-dependent regulation of ghrelin and nesfatin-1 in intestine and hepatopancreas. This study indicates that macronutrients modulate ghrelin and NUCB2/nesfatin-1, and suggests a role for these hormones in the digestive process and nutrient metabolism.

1. Introduction

Ghrelin is a 28-amino-acid peptide mainly synthesized by the gut (Sánchez-Bretaño et al., 2015b). It is a potent orexigenic peptide, as demonstrated in several fishes (Kang et al., 2011; Matsuda et al., 2006; Unniappan et al., 2004) and mammals (Asakawa et al., 2001; Sugino et al., 2004; Tschöp et al., 2000; Wren et al., 2000). Plasma ghrelin in fish, as well as its gene expression were previously measured in response to fasting, feeding and food composition (Blanco et al., 2016a; Jönsson et al., 2007; Riley et al., 2009). From these studies, it seems that ghrelin, while regulating food intake, is in turn modulated by dietary frequency and macronutrients (Horvath et al., 2001; Riley et al., 2009a, b; Velasco et al., 2016). Nesfatin-1 is a peptidyl hormone produced by the N-terminal cleavage of its precursor nucleobindin-2 (NUCB2), encoded by *nucb2* gene (Oh-I et al., 2006). In fish, *nucb2/nesfatin-1* is abundant in many tissues including the brain, pituitary, hepatopancreas, gonads and gastrointestinal tract (Gonzalez et al., 2010; Lin et al., 2014). This peptide reduces food intake after central or peripheral administration in mammals (García-Galiano et al., 2010b; Goebel et al., 2011; Gonzalez et al., 2012; Mortazavi et al., 2015; Oh-I et al., 2006; Shimizu et al., 2009; Sugino et al., 2004; Tang-Christensen et al., 2004) and fish (Gonzalez et al., 2010; Kerbel and Unniappan, 2012), which supports an anorexigenic role for this hormone. It was suggested that both peptides have opposing effects on the regulation of energy balance in fish (Kerbel and Unniappan, 2012).

In mammals, diet composition regulate ghrelin (Beck et al., 2002; Beck and Richy, 2008; Gomez et al., 2012; Handjieva-Darlenska and Boyadjieva, 2009; Kinzig et al., 2007; Wang et al., 2012) and nesfatin-1 (Mohan et al., 2014). We reported that the amount of carbohydrates, protein and lipids present in the diet modulates the expression of *preproghrelin* and *nucb2-nesfatin-1 in vivo* (Blanco et al., 2016b). However, whether components of macronutrients act directly on gut and hepatopancreas to modulate appetite regulatory peptides remain poorly understood. In teleosts, data suggest that endogenous glucose instead of dietary glucose is used as the principal source of energy (Moon, 2001). Moreover, the regulation of ghrelin by glucose found

* Correspondence to: L.F. Canosa, Laboratorio de Neuroendocrinología Molecular, IIB-INTECH, sede Chascomús, CONICET-Universidad, Nacional de San Martin, Av. Intendente Marino Km 8.2, CC 164 (7130) Chascomús, Prov. de Buenos Aires, Argentina.
** Correspondence to: S. Unniappan, Department of Veterinary Biomedical Sciences, Laboratory of Integrative Neuroendocrinology, Western College of Veterinary Medicine, University of Saskatchewan, 52 Campus Drive, Saskatoon, Saskatchewan S7N 5B4, Canada.
Email addresses: jbertucci@intech.gov.ar (J.I. Bertucci); ayelenmelisablanco@ucm.es (A.M. Blanco); lcanosa@intech.gov.ar (L.F. Canosa); suraj.unniappan@usask.ca (S. Unniappan)

http://dx.doi.org/10.1016/j.cbpa.2017.01.006
1095-6433/

in tilapia indicates that dietary glucose may be acting as a signal of metabolic status in fish rather than an energy source (Riley et al., 2009a, b). To date, no data is available on the effects of glucose on NUCB2/nesfatin-1. The protein composition of diet is fundamental to the growth and development of both fish and mammals. Arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine are essential for fish (Akiyama et al., 1997; Tibaldi and Kaushik, 2005). Although several reports have demonstrated the relationship between dietary protein level and food intake in fish (Borlongan and Coloso, 1993; Cowey, 1992; Green and Hardy, 2002; Nunes et al., 2014), the regulation of gastric peptides by amino acid composition of food remains unclear. The role of dietary fatty acids and their saturation levels on ghrelin and NUCB2/nesfatin-1 expression are poorly understood in both fish and mammals. Velasco et al. (2016) found evidence of a possible role of ghrelin on the modulatory effect that fatty acids exert on food intake in rainbow trout. In the case of nesfatin-1, recent data from our group suggest that an increase in the proportion of dietary unsaturated fatty acids leads to a decrease in *NUCB2/nesfatin-1* gene expression in pejerrey larvae (unpublished observations). Also, the presence of both peptides in the hepatopancreas (Jönsson, 2013; Kang et al., 2011) suggest functions for these peptides in this organ.

This research aimed to answer two important questions. Do nutrient components directly modulate the gene and protein expression of ghrelin and NUCB2/nesfatin-1 in the gastrointestinal tract and hepatopancreas of fish? Do the levels of fatty acid saturation affect the expression of this two appetite regulatory peptides? As a first approach to answer these questions, the presence and cellular localization of ghrelin and NUCB2/nesfatin-1 in goldfish hepatopancreas were characterized by immunohistochemistry. Second, an organ culture experiment using goldfish intestine and hepatopancreas treated with different concentrations of glucose, the essential amino acid L-tryptophan or different fatty acids varying in length and unsaturation level was performed.

2. Material and methods

2.1. Fish

Goldfish (*Carassius auratus*; comet variety), with a body weight of 5 ± 1 g (for immunohistochemistry) or 32 ± 8 g (for organ culture), were acquired from a commercial supplier (Aquatic Imports, Calgary, AB, Canada) and maintained at 20 ± 2 °C and a 12 h:12 h dark-light cycle photoperiod in 300 L tanks. Fish were fed *ad libitum* once daily (10:00 am) on a commercial diet for goldfish (Goldfish granules, Aqueon, Franklin, WI, USA). All studies using fish strictly followed the Canadian Council of Animal Care guidelines, and protocols were approved by the Animal Research Ethics Board of the University of Saskatchewan (Protocol Number 2012-0082).

2.2. Immunohistochemistry (IHC)

Goldfish (n = 3), which were fasted for 24 h, were sacrificed by spinal dissection and then immersed in 4% paraformaldehyde (PFA) diluted in 0.1 M phosphate-buffered saline (PBS, pH 7.4) overnight. The following day, the hepatopancreas was removed and post-fixed for 3 h in a fresh 4% PFA solution. Tissue was processed (dehydrated and embedded in paraffin) at the Prairie Diagnostic Services, University of Saskatchewan. Paraffin blocks were then sectioned at 7 μm thickness using a rotary microtome, and transversal sections were mounted onto superfrost slides. The protocol for IHC was performed as previously described (Diotel et al., 2011) with slight modifications. Sections were deparaffinized with xylene (twice, for 5 min each) and rehydrated through a series of graded ethanol (100% ethanol for 2 min twice, 95% ethanol, 85% ethanol, 70% ethanol, 50% ethanol and 30% ethanol for 30 s once each). Sections were then washed with 0.85% NaCl (twice for 10 min) and 0.1 M PBS (once for 5 min), and blocked in 0.1 M PBS containing 0.5% of bovine serum albumin for 45 min before being incubated overnight with a mixture of primary antibody against ghrelin and primary antibody against nesfatin-1 (rabbit anti-ghrelin, Catalog # H-031-31, Phoenix Pharmaceuticals, Burlingame, CA, USA; mouse anti-nesfatin-1, Catalog # ALX-804-854-C100, Enzo Life Sciences, Brockville, ON, Canada) both diluted 1:200, at room temperature. Ghrelin and nesfatin-1 antibodies detect both precursor (preproghrelin or NUCB2, respectively) and processed ghrelin or nesfatin-1. Preproghrelin and NUCB2 antibodies were developed in rabbit and mouse, respectively, using the corresponding rat peptidic sequences (ghrelin: GSSFLSPEHQKAQQRKESKKPPAKLQPR; nesfatin-1: PIDVDKTKVHNVEPV ESARIEPPDTGLYYDEYLKQVIEVLETDPHFREKLQKADIEEIRSGRLSQELDLVSHKVRTRLDEL) as immunogen. Preabsorption controls using goldfish synthetic ghrelin and nesfatin-1 were performed to control for the binding of these heterologous antibodies to the endogenous peptides. Despite this, a small degree of non-specificity could be ruled out, so the positive immunoreactivity signal is mentioned here as ghrelin-like and NUCB2/nesfatin-1-like, instead of ghrelin and NUCB2/nesfatin-1. The following day, sections were washed twice in PBS and once in 0.2% Triton PBS for 10 min each and subsequently incubated with a mixture of Texas Red anti-rabbit IgG (Vector Laboratories, Burlington, ON, Canada) and FITC anti-mouse IgG (Abcam, Toronto, ON, Canada), both diluted 1:2000, for 1 h at room temperature. Separate sets of slides were treated only with the secondary antibodies (negative controls). Preabsorption controls were carried out by an overnight incubation of the slides with mixtures of goldfish ghrelin or nesfatin-1, at a ratio 1:10 (antibody:peptide). All primary and secondary antibodies were diluted in antibody diluent reagent (Dako, Mississauga, ON, Canada). After incubation with secondary antibodies, slides were washed twice in PBS and once in 0.2% Triton PBS for 5 min. Finally, sections were mounted using VECTASHIELD Mounting Medium containing 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlington, ON, Canada). Slides were assessed using a Nikon Eclipse Ti-Inverted fluorescence microscope (Nikon Instruments, Melville, NY, USA), and images were captured using a Nikon DS-Qi1 MC camera. Images were analyzed using the NiS Elements Basic Research Imaging Software and adjusted linearly for light and contrast before being assembled on plates using Photoshop CS6 (Adobe Systems Inc., San Jose, CA, USA).

2.3. Organ culture experiment

2.3.1. Reagents

All macronutrient component concentrations were chosen based on the doses used *in vitro* in mouse cells by Mohan et al., 2014, and *in vivo* in goldfish by Blanco et al., 2016a. We tested glucose to represent the effect of carbohydrates on fish metabolism, and L-tryptophan to represent the effect of the essential amino acids. Additionally, to know whether the fatty acids influence ghrelin and NUCB2/nesfatin-1 and if their unsaturation level plays a role in such modulation, we tested oleic acid, linolenic acid (LNA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Glucose (Fisher Scientific, Ottawa, ON, Canada) and L-tryptophan (Sigma-Aldrich, Oakville, ON, Canada) were reconstituted in low-glucose (5.6 mM) Dulbecco's Modified Eagle Medium (DMEM) supplemented with 44 mM sodium bicarbonate, 1% penicillin-streptomycin and 0.05% gentamicin (DMEM +) at a stock concentration of 100 mM. Oleic acid,

LNA, EPA and DHA (Sigma-Aldrich) were reconstituted at a stock concentration of 10 mM. All stock solutions were diluted in DMEM + to reach the required experimental concentrations just before use. Low glucose-DMEM medium was used in all cultures in order to avoid potential masking effects of high glucose levels on the macronutrients effect on ghrelin and NUCB2/nesfatin-1gene expression.

2.3.2. Culture conditions and experimental assays

Organ culture was performed as previously described for goldfish (Sánchez-Bretaño et al., 2016) with slight modifications. Goldfish (n = 6) fasted for 24 h were sacrificed by spinal dissection, and the anterior intestine (from the end of the intestinal bulb to the J-loop) and hepatopancreas were quickly removed under sterile conditions. Intestines were cut into fragments of approximately 1–2 mm width and immersed in DMEM supplemented 10% penicillin-streptomycin and 0.5% gentamicin for 1 min, before being distributed in different wells of sterile uncoated culture 24-well multidish plates (Corning®, Tewksbury, MA, USA) (20 mg tissue/well) containing 1 mL of DMEM +. Hepatopancreas were disrupted with a sterile scalpel and portions of tissue (20 mg tissue/well) were placed directly in the culture plates, also in wells containing 1 mL of DMEM +. Plates were preincubated for 2 h at 23 °C under an atmosphere of 5% CO_2 and 95% O_2 for stabilization. Then, medium was replaced by 1 mL of fresh DMEM + alone (control) or containing either glucose (25, 50 or 100 mM), L-tryptophan (1, 10 or 50 mM), oleic acid (1, 10 or 100 μM), LNA (1, 10 or 100 μM), EPA (1, 10 or 100 μM) or DHA (1, 10 or 100 μM). Plates were designed so that each of it contains 6 control wells and 18 wells treated with a single drug (6 wells per concentration). In each plate, the 6 replicates per experimental group contain tissue from a different fish. Besides, each plate was generated in duplicate, one of which was incubated for 2 and the other for 4 h. At the end of each incubation time, intestine and hepatopancreas samples were collected, quickly frozen in liquid nitrogen and stored at − 80 °C until total RNA or protein was extracted.

2.4. Real-time quantitative PCR (RT-qPCR)

Total RNA was isolated using Ribozol RNA Extraction Reagent (aMReSCO, Toronto, ON, Canada) following the protocol provided by the manufacturer. RNA purity was validated by optical density (OD) absorption ratio (OD 260 nm/280 nm) using a NanoDrop 2000c (Thermo, Vantaa, Finland). An aliquot of 1 μg of total RNA was reverse transcribed into cDNA in a 20 μL reaction volume using iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Mississauga, ON, Canada) according to the manufacturer's instructions. RT-qPCRs were performed using iQ SYBR Green Supermix (Bio-Rad, Mississauga, ON, Canada). The specific primer sequences used for target genes *preproghrelin* and *nucb2/nesfatin-1*, and reference gene *β-actin* are shown in Table 1. Primers were purchased from IDT (Toronto, ON, Canada). Each sample was run in duplicate using a 96-well plate (Axygen, Union City, CA, USA) loaded with 1 μL of cDNA and 500 nM of each forward and reverse primer in a final volume of 10 μL. Each PCR run included a standard curve for the gene tested made of four points of a serial dilution (1:1; 1.3; 1:9 and 1:27). Negative control was carried out by adding water instead of cDNA. RT-qPCR cycling conditions consisted of an initial step of 95 °C for 3 min, and 35 cycles of 95 °C for 10 s and 56.6 °C (*preproghrelin*) or 60 °C (*nucb2/nesfatin-1* and *β-actin*) for 30 s. A melting curve was systematically monitored (temperature gradient at 0.5 °C/5 s from 65 to 95 °C) at the end of each run to confirm specificity of the amplification reaction. The efficiency of the amplification for all genes studied was 95–100%. All runs were performed using a CFX Connect Real-Time System (Bio-Rad, Mississauga, ON, Canada). The $2 - \Delta\Delta Ct$ method (Livak and Schmittgen, 2001) was used to determine the relative mRNA expression.

Table 1
Primers used for quantifying gene expression by RT-qPCR.

Gene	GenBank accession number	Primer sequence (5′ to 3′)	Product size (bp)
Preproghrelin	AF454389.1	F: ATTCAGAGTGTTGTCGTA R: AGGAAAGAGCACATAAGA	103
Nucb2/nesfatin-1	HM065567.1	F: AGTCTCCCCAGAATGTGGAC R: CATCCAGCTTGGTTCTCACA	186
β-Actin	AB039726.2	F: CAGGGAGTGATGGTTGGCA R: AACACGCAGCTCGTTGTAGA	168

F, Forward primer; R, Reverse primer.

2.5. Western blot analysis

Ghrelin and NUCB2/nesfatin-1 protein levels in cultured intestine and hepatopancreas were quantified by Western blot. Only selected samples that were found to have notable effects on mRNA expression after glucose, tryptophan and fatty acids administration were used for protein quantification. Tissues (n = 4 fish) were homogenized in T-PER tissue protein extraction reagent (Thermo Fisher Scientific, Waltham, MA, USA), and proteins were extracted according to the manufacturer's instructions and quantified by Bradford assay. Western blot protocol was performed as previously described (Ramesh et al., 2015). The samples (containing 30 μg protein) were prepared in 1 × Laemmli buffer containing 0.2% 2-mercaptoethanol (Bio-Rad) and subsequently were boiled at 95 °C for 10 min. Then, the whole sample volume was loaded and electrophoresed in 8–16% Mini-PROTEAN® TGX™ precast protein gels (Bio-Rad). Precision plus protein™ Dual Xtra standards (Bio-Rad) was used as molecular weight marker. Following electrophoresis, proteins were transferred to a 0.2 μm pore-size nitrocellulose membrane (Bio-Rad) using the Trans-Blot® Turbo™ transfer system (Bio-Rad), and membrane was blocked in 1 × RapidBlock™ solution (aMReSCO). Detection of target proteins was performed by overnight incubation of membrane in 1 × RapidBlock™ solution containing specific primary antibody (1:500 dilution; mouse anti-ghrelin, Catalog # ab57222, Abcam; mouse anti-nesfatin-1, Catalog # ALX-804-854-C100, Enzo Life Sciences). Vinculin protein was used for normalization and was detected using rabbit antiserum directed against mouse vinculin (1:2000 dilution; Catalog # ab129002, Abcam). As secondary antibody, goat anti-mouse or anti-rabbit IgG (H + L) HRP conjugate (Bio-Rad) diluted 1:2000 were used. For protein visualization the membrane was incubated for 5 min in Clarity™ Western ECL substrate (Bio-Rad) and imaged using ChemiDoc™ MP imaging system (Bio-Rad) with chemiluminescence detection. Blot images were plotted using ImageJ software and band density of vinculin was used to normalize protein density.

2.6. Statistical analysis

Data were analyzed by one-way ANOVA, followed by *post hoc* Student-Newman-Keuls test at a significance level of $p < 0.05$. Data that failed to pass homogeneity tests were log-transformed and

re-tested. All tests were performed using Infostat Version 2008 (JA di Rienzo et al., 2013) software.

3. Results

3.1. Co-localization of ghrelin and NUCB2/nesfatin-1 in goldfish hepatopancreas

Ghrelin and NUCB2/nesfatin-1 were detected in the cytoplasm of cells in the goldfish hepatopancreas (Fig. 1 A, B). Immunoreactivity of each peptide is distributed through the liver and may include pancreatic cells. As seen in Fig. 1 A and B, most of the NUCB2/nesfatin-1 signal appears in cells that are surrounding a centrilobular vein, in a structure that seems to be a hepatic lobe. In this area, ghrelin signal is stronger than the one for NUCB2/nesfatin-1. Moreover, ghrelin immunoreactivity is widely distributed around the hepatopancreas compared with NUCB2/nesfatin-1. Also, we found that some of the cells co-localize both peptides (Fig. 1 C). No staining was detected in both negative and preabsorption controls (Fig. 1 D, E, F).

3.2. Glucose modulates preproghrelin and nucb2/nesfatin-1 mRNA expression in a concentration- and time-dependent manner

In the intestine, the highest glucose concentration led to a statistically significant decrease in *preproghrelin* expression after 2 h of treatment, whereas at 4 h, 50 mM and 100 mM significantly increased its expression (Fig. 2 A). On the other hand, the expression of *nucb2/nesfatin-1* significantly decreased compared to control in all glucose treatments after 2 and 4 h (Fig. 2 B). In the hepatopancreas, 100 mM of glucose led to a significant decrease in *preproghrelin* expression compared to the control group (Fig. 2 C), while no changes were observed in *nucb2/nesfatin-1* mRNA expression regardless of the dose and treatment duration (Fig. 2 D).

3.3. L-Tryptophan downregulates preproghrelin mRNA expression and time-dependently modulates nucb2/nesfatin-1 mRNA expression.

In the intestine, *preproghrelin* expression significantly decreased compared to control after 2 h of treatment with all concentrations of L-tryptophan, while no changes were observed after 4 h (Fig. 3 A). *Nucb2/nesfatin-1* expression showed a statistical decrease after 2 h of exposure to 10 mM and 50 mM of L-tryptophan, but was significantly upregulated after 4 h in the group treated with 50 mM (Fig. 3 B). In the hepatopancreas, L-tryptophan led to a significant decrease in *preproghrelin* expression at 4 h, while no differences were observed between control and treated groups at 2 h (Fig. 3 C). In the case of *nucb2/nesfatin-1*, its mRNA expression significantly decreased in groups treated with 10 mM and 50 mM L-tryptophan for 2 h, but significantly increased after 4 h of treatment with 50 mM L-tryptophan (Fig. 3 D).

3.4. Fatty acids predominantly downregulate the intestinal preproghrelin and nucb2/nesfatin-1 mRNA expression

As shown in Fig. 4 A and B, 10 μM oleic acid treatment led to a significant increase in both *preproghrelin* and *NUCB2/nesfatin-1* mRNA expression at 2 h. After 4 h of treatment, 1 μM oleic acid significantly increased the expression of *preproghrelin* (Fig. 4 A). *Nucb2/nesfatin-1* mRNA levels were significantly decreased after 2 h of treatment with 1 μM and 100 μM of oleic acid, and after 4 h of exposure to all concentrations tested (Fig. 4 B). No changes in *preproghrelin* expression were observed after 2 and 4 h of exposure to LNA, except in the group treated with 10 μM LNA during 4 h in which a significant decrease in mRNA levels was observed (Fig. 4 C). On the other hand, all doses of LNA significantly decreased the *NUCB2/nesfatin-1* mRNA expression compared to control group after 2 and 4 h of treatment (Fig. 4 D). All doses of EPA led to a signif-

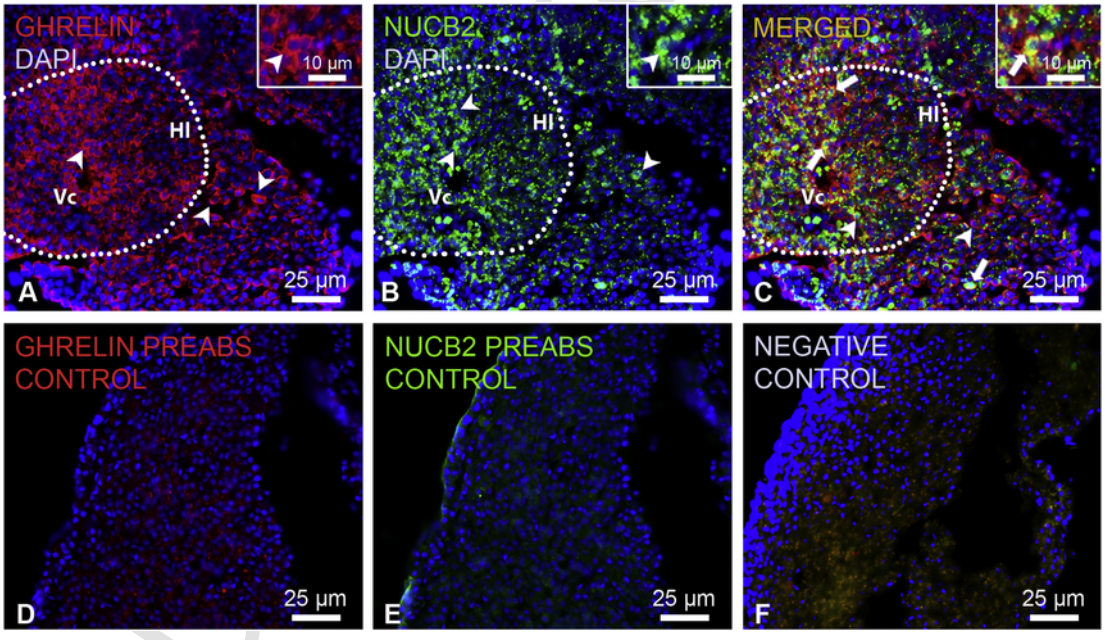

Fig. 1. Co-localization of ghrelin and NUCB2/nesfatin-1 in goldfish hepatopancreas. Immunoreactivity to ghrelin (red, panel A) and NUCB2/nesfatin-1 (green panel B) is localized in the cytoplasm of hepatopancreas cells. Several cells co express both ghrelin and NUCB2 (C). Either preabsorption with corresponding synthetic peptide or absence of corresponding primary antibody yield non immunoreactivity signal (D, E, F). All images are merged with DAPI showing nuclei in blue. Arrowheads indicate cells stained for either ghrelin or NUCB2 and solid arrows show cells that show co-localization of both ghrelin and NUCB2/nesfatin-1. In panels (B), (C) and (E) NUCB2 labels refer to both NUCB2 and nesfatin-1 proteins. Dash line encloses the hepatic lobe area (Hl). Vc indicates the centrilobular vein. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

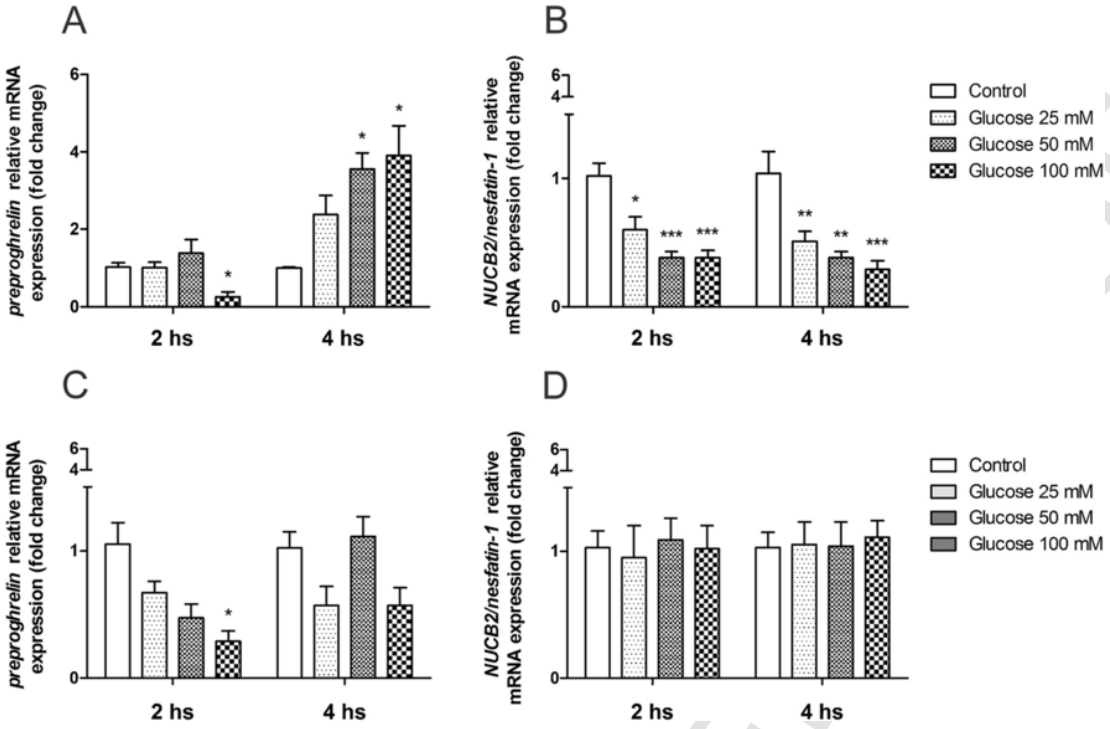

Fig. 2. *Preproghrelin* and *nucb2/nesfatin-1* mRNA expression in intestine (A, B) and hepatopancreas (C, D) of goldfish after 2 h and 4 h of 25 mM, 50 mM and 100 mM glucose treatments. Data obtained by RT-qPCR are shown as mean + SEM (n = 6 fish). Asterisks denote statistical differences between control and treated groups assessed by one way ANOVA follow by Student-Newman-Keuls post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001).

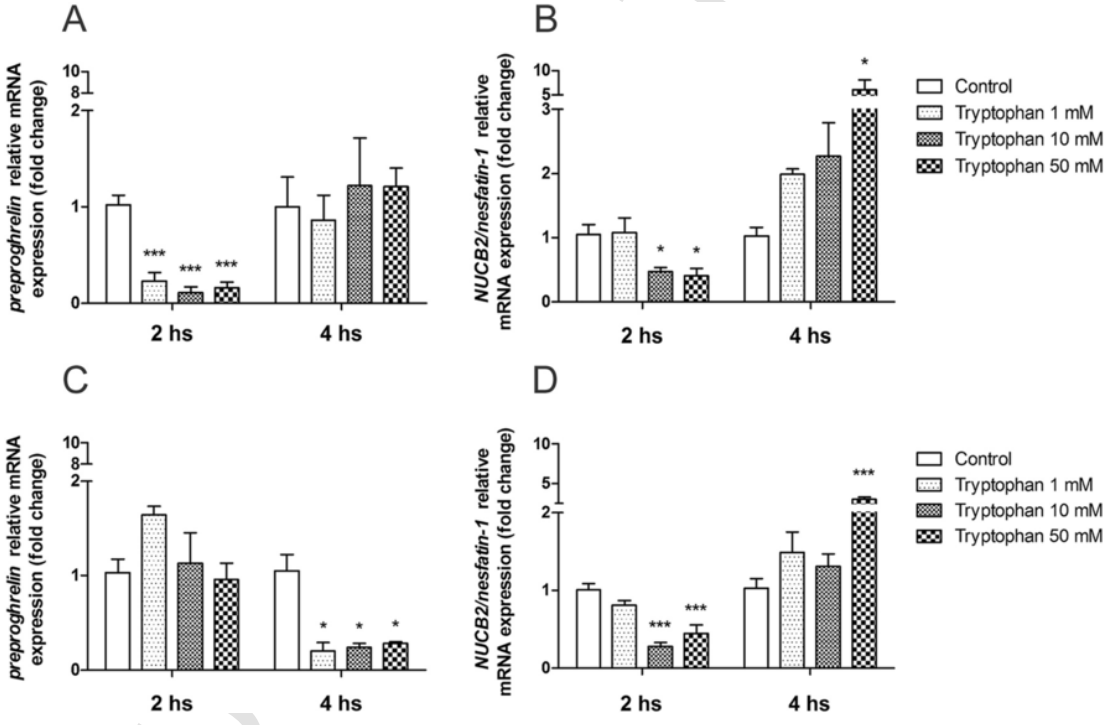

Fig. 3. *Preproghrelin* and *nucb2/nesfatin-1* mRNA expression in intestine (A, B) and hepatopancreas (C, D) of goldfish after 2 h and 4 h of 1 mM, 10 mM and 50 mM L-tryptophan treatment. Data obtained by RT-qPCR are shown as mean + SEM (n = 6 fish). Asterisks denote statistical differences between control and treated groups assessed by ANOVA and Student-Newman-Keuls post-hoc test (*p < 0.05, ***p < 0.001).

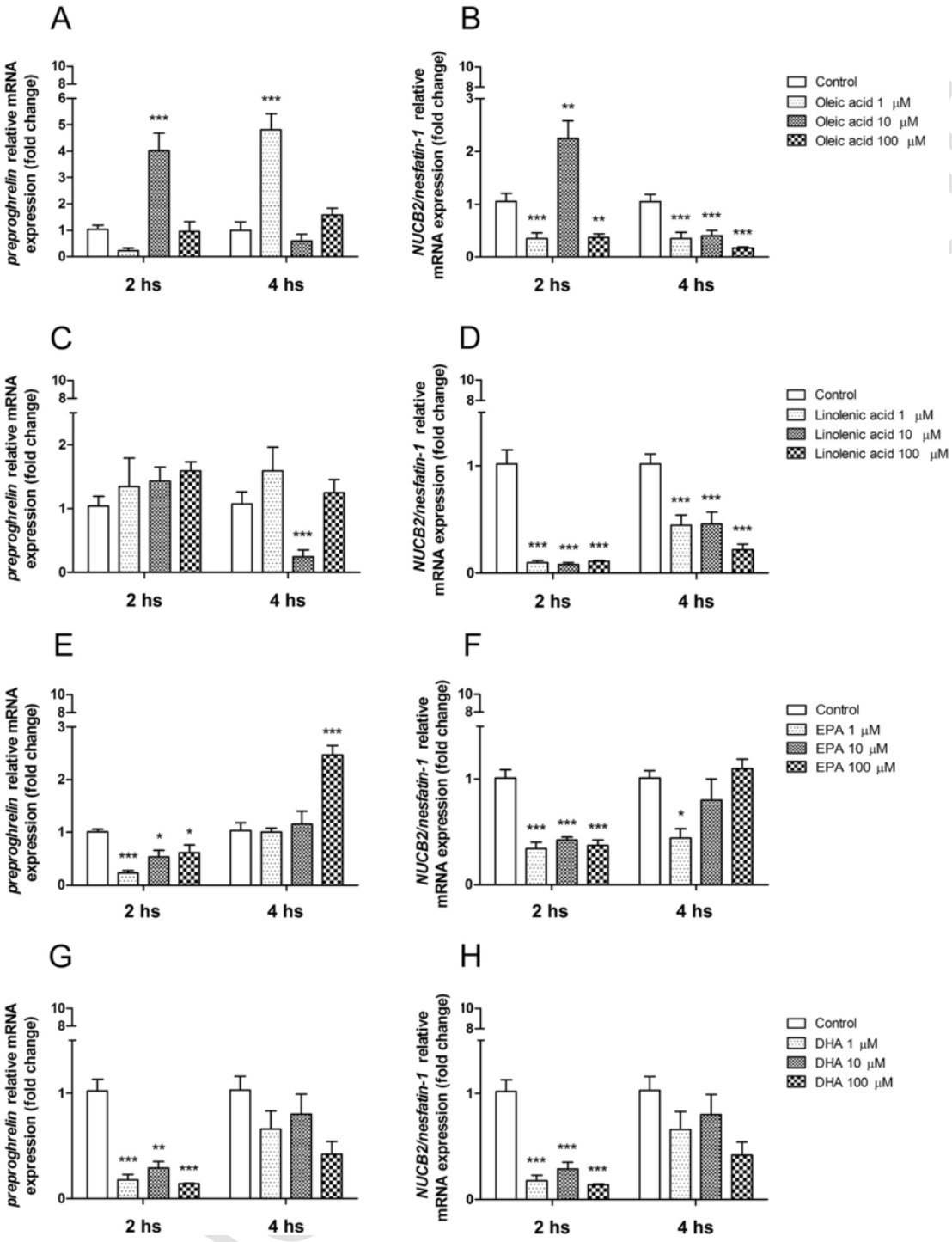

Fig. 4. *Preproghrelin* and *nucb2/nesfatin-1* mRNA expression in intestine after 2 h and 4 h of treatment with 1 mM, 10 mM and 100 mM oleic acid (A, B), linolenic acid (LNA; C, D), eicosapentanodioic acid (EPA; E, F) and docosahexanodioic acid (DHA; G, H). Data obtained by RT-qPCR are shown as mean + SEM (n = 6 fish). Asterisks denote statistical differences between control and treated groups assessed by ANOVA and Student-Newman-Keuls post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001).

icant decrease in both *preproghrelin* and *nucb2/nesfatin-1* mRNA expression after 2 h of treatment (Fig. 4 E, F). The 100 μM concentration of LNA significantly increased the expression of *preproghrelin*, whereas the 1 μM concentration significantly decreased the *NUCB2/nesfatin-1* expression at 4 h (Fig. 4 E, F). Exposure to all doses of DHA resulted in a significant decrease in both *preproghrelin* and *nucb2/nesfatin-1* mRNA expressions after 2 h, while no significant changes were observed after 4 h (Fig. 4 G, H).

3.5. Fatty acids modulate preproghrelin and nucb2/nesfatin-1 mRNA expression in cultured hepatopancreas in a time- and concentration-dependent manner

Oleic acid (10 μM) significantly increased the *preproghrelin* and *nucb2/nesfatin-1* mRNA expression after 2 h of treatment. All the other concentrations of oleic acid tested led to a significant decrease

in the *preproghrelin* mRNA expression, and did not alter the expression of *NUCB2/nesfatin-1* after either 2 or 4 h of treatment (Fig. 5 A, B). As shown in Fig. 5 C and D, LNA significantly decreased *preproghrelin* expression after 2 and 4 h of treatment. *Nucb2/nesfatin*-1 mRNA expression remained unaltered after 2 h of exposure to all LNA concentrations tested, while after 4 h the 100 μM LNA dose significantly increased the *Nucb2/nesfatin*-1 mRNA expression.

Treatments with 10 μM and 100 μM EPA led to a significant decrease in *preproghrelin* expression and a significant increase in *nucb2/nesfatin*-1 expression at 2 h (Fig. 5 E, F). However, all concentrations of EPA significantly increased the expression of *preproghre-*

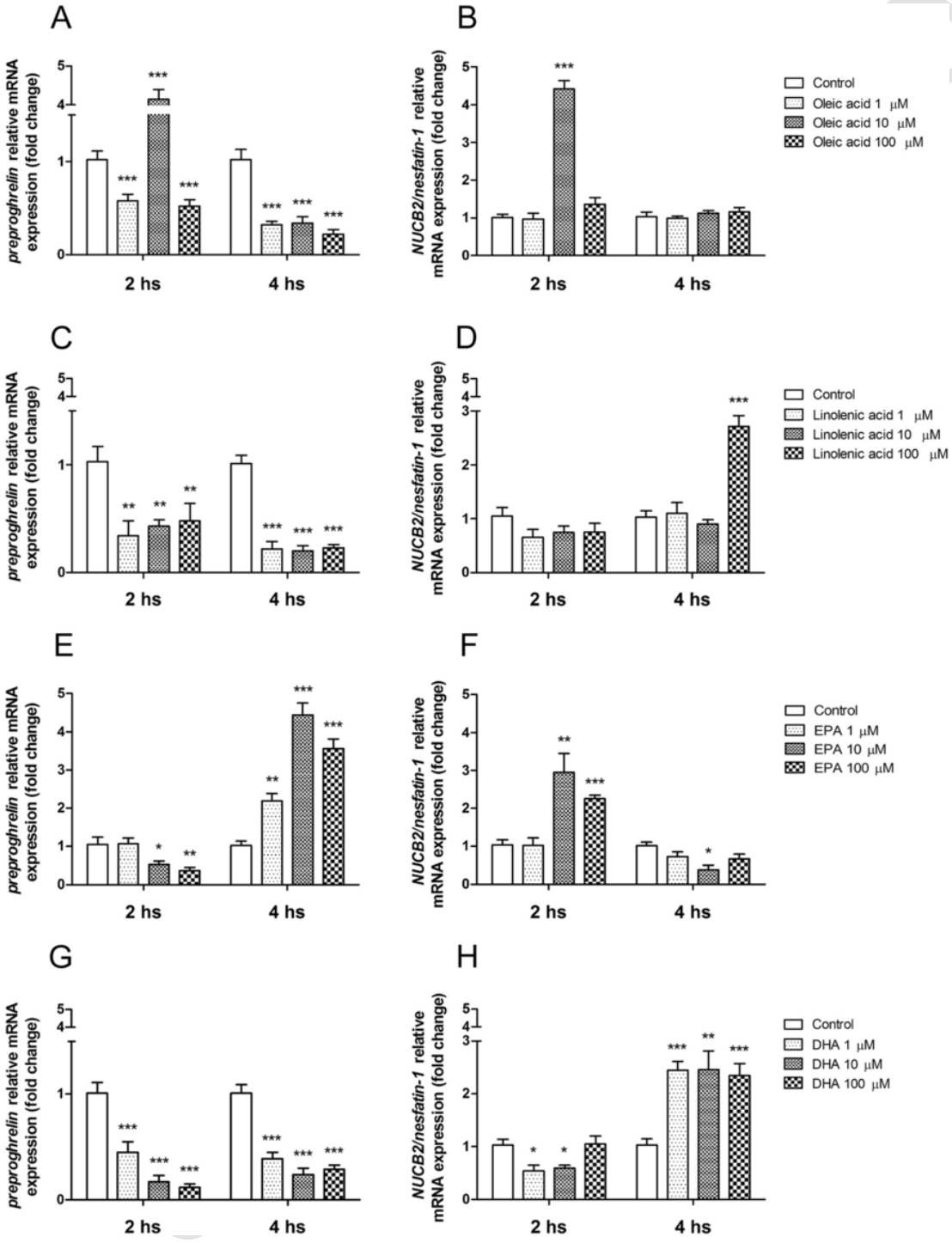

Fig. 5. *Preproghrelin* and *nucb2/nesfatin-1* mRNA expression in hepatopancreas after 2 h and 4 h of treatment with 1 mM, 10 mM and 100 mM oleic acid (A, B), linolenic acid (LNA; C, D), eicosapentanodioic acid (EPA; E, F) and docosahexanodioic acid (DHA; G, H). Data obtained by RT-qPCR are shown as mean + SEM (n = 6 fish). Asterisks denote statistical differences between control and treated groups assessed by ANOVA and Student-Newman-Keuls post-hoc test (*p < 0.05, **p < 0.01, ***p < 0.001).

lin at 4 h (Fig. 5 E), while the 10 μM concentration significantly decreased the *nucb2/nesfatin*-1 mRNAs levels after 4 h of treatment (Fig. F). As can be seen in Fig. 5 G, all DHA concentrations led to a significant decrease in the *preproghrelin* mRNA expression after 2 and 4 h of treatment. *Nucb2/nesfatin*-1 mRNA expression was significantly decreased after 2 h of treatment with 1 μM and 10 μM of DHA, and was significantly increased with all doses of DHA after 4 h of treatment (Fig. 5 H).

3.6. Macronutrients alter ghrelin and nesfatin-1 protein expression

In the intestine, the expression of preproghrelin significantly decreased after 2 h of treatment with 100 μM DHA. No changes were observed with 50 mM tryptophan and 100 μM EPA treatments (Fig. 6A). After 4 h of treatment, preproghrelin levels were significantly higher than control in the group treated with 100 mM glucose (Fig. 6B). As is shown in Fig. 6 C, no changes were detected in NUCB2

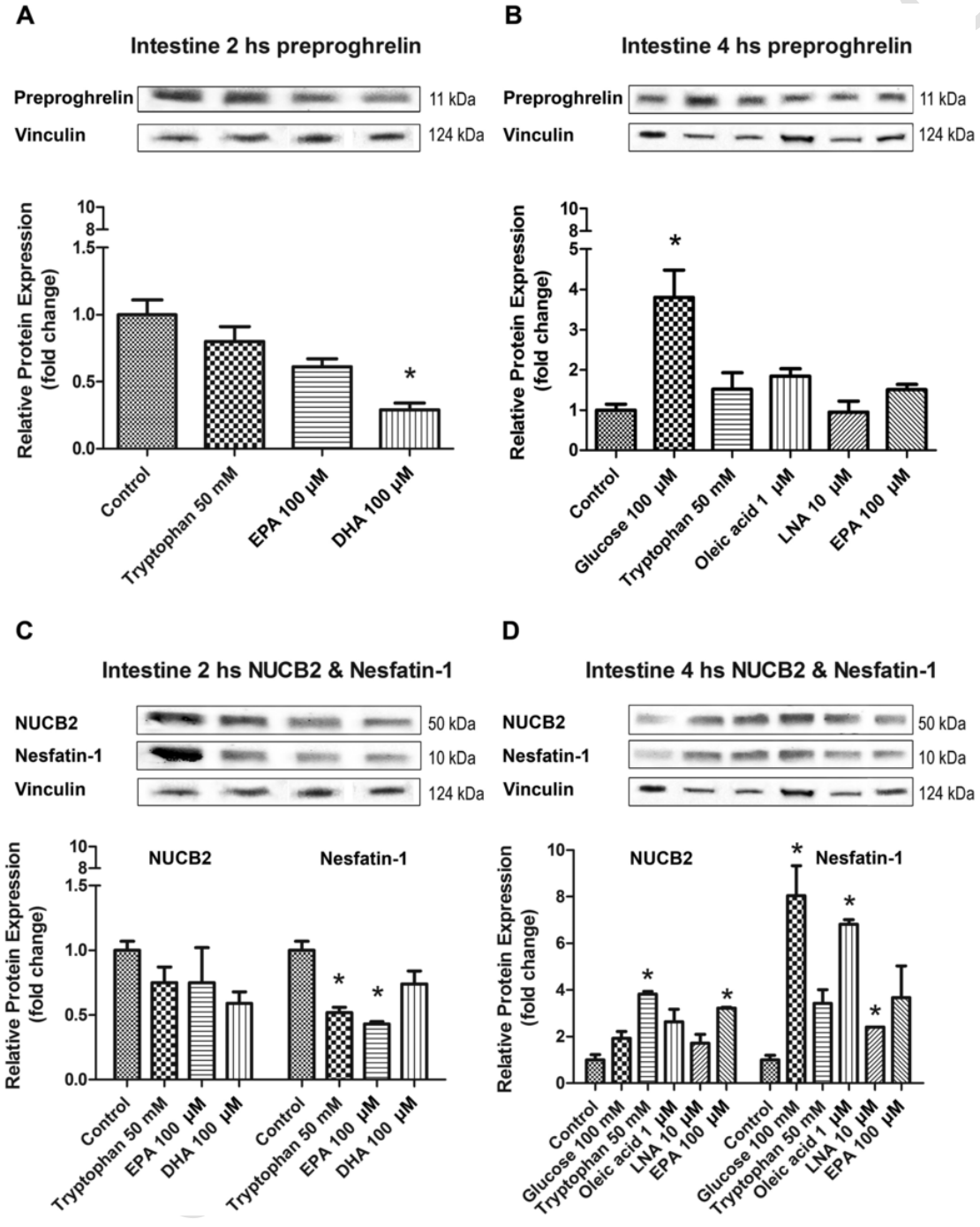

Fig. 6. Ghrelin (A, B) and NUCB2/nesfatin-1 (C, D) protein expression in intestine after 2 h and 4 h of treatment with different macronutrients. Data obtained by band densitometry are shown as mean + SEM (n = 4 fish). Asterisks denote statistical differences between control and treated groups assessed by ANOVA and Student-Newman-Keuls post-hoc test (*$p < 0.05$).

levels after 2 h of treatment with the different macronutrients in intestine. On the other hand, the protein expression of nesfatin-1 significantly decreased compared to control in those groups treated with 50 mM L-tryptophan and 100 μM EPA during 2 h. After 4 h of treatment, a statistically significant high expression of NUCB2 was observed after exposure to 50 mM L-tryptophan and 100 μM EPA (Fig. 6 D). A significant increase in nesfatin-1 levels was detected in those groups treated with 100 mM glucose and 1 μM oleic acid, while a decrease was observed after treatment with 10 μM LNA (Fig. 6 D).

In the hepatopancreas, preproghrelin protein expression was significantly higher compared to control in the group treated with 10 μM oleic acid during 2 h (Fig. 7 A). After 4 h, the group treated with 100 μM DHA showed a significant decrease in preproghrelin levels (Fig. 7 B). The expression of NUCB2, but not nesfatin-1, signifi-

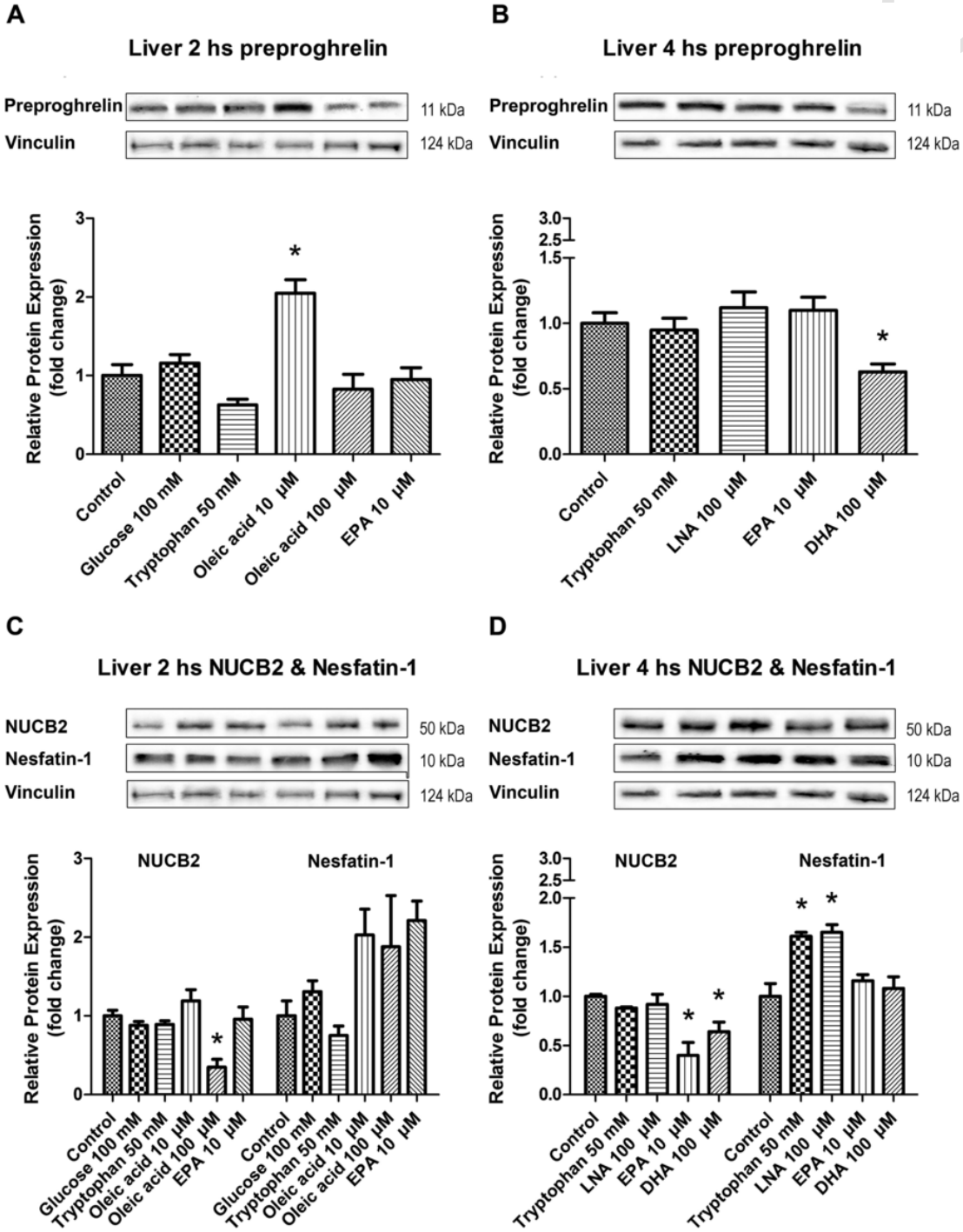

Fig. 7. Ghrelin (A, B) and NUCB2/nesfatin-1 (C, D) protein expression in hepatopancreas after 2 h and 4 h of treatment with different macronutrients. Data obtained by band densitometry are shown as mean + SEM (n = 4 fish). Asterisks denote statistical differences between control and treated groups assessed by ANOVA and Student-Newman-Keuls post-hoc test (*$p < 0.05$).

cantly decreased after 2 h of treatment with 100 μM oleic acid (Fig. 7 C). A significant decrease in NUCB2 levels was observed in those groups treated with 10 μM EPA and 100 μM DHA after 4 h (Fig. 7 D). Treatments with 50 mM L-tryptophan and 100 μM LNA led to a significant increase in nesfatin-1 expression after 4 h (Fig. 7 D).

4. Discussion

Previously, we found that diets containing varying amounts of macronutrients modulate *preproghrelin* and *nucb2/nesfatin-1* mRNA expression *in vivo* in central and peripheral tissues of goldfish (Blanco et al., 2016a). Here, we investigated if macronutrients directly regulate the mRNA and protein expression of preproghrelin and NUCB2/nesfatin-1 in peripheral tissues. To achieve this, goldfish intestine and hepatopancreas were cultured and treated with glucose, L-tryptophan and different fatty acids. We chose intestine and hepatopancreas, because these two tissues are heavily involved in the regulation of feeding and metabolism, and both ghrelin and nesfatin-1 (Asakawa et al., 2005; Stengel et al., 2010., Gonzalez et al., 2012, Gonzalez et al., 2010; Kaiya et al., 2008) are expressed in these tissues, at least at gene level. This is the first report in which direct effects of macronutrients on feeding regulatory peptides are reported *in vitro* in non-mammalian tissues.

Our first aim was to demonstrate the presence of ghrelin and nesfatin-1 in goldfish hepatopancreas by IHC. The presence of ghrelin has been demonstrated by both PCR and IHC techniques in the intestine of fish (Riley et al., 2009a, b; Sánchez-Bretaño et al., 2015a, b). However, the presence of both peptides in hepatopancreas has only been studied using RT-qPCR (Blanco et al., 2016a; Lin et al., 2014; Mohan and Unniappan, 2013). We provide evidence for the cellular distribution of ghrelin and NUCB2/nesfatin-1 in goldfish liver, showing that both peptides are present in the cytoplasm of hepatopancreatic cells, and that some of these cells co-localize both ghrelin and nesfatin-1. The presence of ghrelin and nesfatin-1 in hepatopancreas, a tissue with a major role in metabolism, supports an involvement of both hormones not only in food intake regulation, but also in metabolism and energy homeostasis (Al Massadi et al., 2014; García-Galiano et al., 2010a; Jönsson, 2013).

We found that macronutrients exert a modulatory effect on *preproghrelin* and *nucb2/nesfatin-1* mRNA and protein expression in both tissues studied, which is time- and concentration-dependent. In some cases, the effects on mRNA and protein expression were opposite. This could be due to the distinct effects of micronutrients on transcription, translation and post-translational processing. In the case of glucose, we observed that, in the intestine, it mainly increased the mRNA and protein expression of preproghrelin and led to a decrease in *nucb2/nesfatin-1* mRNA expression. While no changes in NUCB2 protein expression was observed in intestine treated with glucose, an increase in nesfatin-1 was detected. These results indicate that glucose induces changes not only at the mRNA expression level, but also in the process that regulate the cleavage of nesfatin-1 from its precursor. Taking together, the effects observed suggest a global orexigenic effect exerted by glucose in the intestine, which is the principal tissue responsible for ghrelin production (Kojima et al., 1999) and also show a small synthesis of NUCB2/nesfatin-1 (Lin et al., 2014). An increase in *preproghrelin* mRNA expression, as well as plasma ghrelin levels, in response to glucose was previously reported in the intestine of tilapia (Riley et al., 2009a, b), supporting an orexigenic action of glucose in fish. Additionally, the higher expression of *preproghrelin* found *in vitro* here, and *in vivo* by other authors could support a role of this peptide in the control of glucose metabolism (Dezaki, 2013). In the hepatopancreas, glucose had no effects on preproghrelin and NUCB2/nesfatin-1 mRNA expression. In this tissue, glucose decreases preproghrelin mRNA expression just with the highest dose. This might be explained by the fact that, at least in some fish including carp and goldfish, the adipose tissue instead of the liver seems to play a major role in glucose metabolism (Roy et al., 2003). In mammals, a decrease in plasma ghrelin after intravenous and oral administration of glucose was found (Nakagawa et al., 2002; Shiiya et al., 2002). These results suggest an interaction between ghrelin and insulin in mammals (Blom et al., 2005). A direct relationship between nesfatin-1 and insulin effects on glucose was found in mammals (Gonzalez et al., 2011; Li et al., 2013; Mohan and Unniappan, 2012). Also, an increase of *NUCB2/nesfatin-1* mRNA expression in response to glucose was found *in vitro* in ghrelinoma cells from mouse (Mohan et al., 2014). These results, which contradicts the present work, highlight the major differences regarding glucose metabolism between fish and mammals (Hemre et al., 2002).

The role of dietary proteins in somatic growth of both fish and mammals has been extensively studied and they seem to be the most important factor limiting it (Abi-Ayad and Kestemont, 1994; Lochmann and Phillips, 1994; Perez-Sanchez et al., 1995). Despite that, only limited research has been carried out on the importance of dietary protein levels on food intake (Wang et al., 2012). We aimed to elucidate the role of proteins in the regulation of ghrelin and NUCB2/nesfatin-1, and tested different doses of L-tryptophan, an essential amino acid for fish. In the intestine, we found that L-tryptophan downregulates the expression of *preproghrelin* at 2 h, and that it exerts a different effect on the expression of NUCB2/nesfatin-1 depending on the duration of the treatment. Thus, 2 h of exposure to this nutrient resulted in a decrease in NUCB2/nesfatin-1 mRNA and protein levels, but an increase was observed after 4 h. This is in accordance with previous reports in mammals (Lejeune et al., 2006; Nieuwenhuizen et al., 2009; Veldhorst et al., 2008). In the hepatopancreas, the highest dose of L-tryptophan exerted opposite effects at 4 h, where it downregulated *preproghrelin* mRNA expression and upregulated NUCB2/nesfatin-1 mRNA and protein levels. Unpublished observations from our group demonstrate that ghrelin co-localizes hepatopancreatic enzymes, including trypsin that is involved in protein metabolism (Lauff and Hofer, 1984). Both ghrelin and nesfatin-1 also modulate digestive enzymes (Blanco et al., 2017). Dietary amino acids, such as tryptophan, could regulate the actions of these enzymes through ghrelin and nesfatin-1.

We then determined how the length and saturation level of fatty acids could affect the expression of ghrelin and nesfatin-1. For this, we tested four different fatty acids: oleic acid (18:1n − 9), which contains 18 carbon atoms and 1 double bond; LNA (18:3n − 3), which has 18 carbon atoms and 3 double bonds; EPA (20:5n − 3), which contains 20 carbon atoms and 5 double bonds; and DHA (22:6n − 3), which contains 22 carbon atoms and 6 double bonds. It is known that herbivorous and omnivorous fish that are in the bottom of the food chain has the capability to add unsaturated and elongated fatty acids in order to obtain polyunsaturated fatty acids (PUFAs; such as EPA and DHA) from their precursors including LNA (Castro et al., 2012; Vagner and Santigosa, 2011; Zheng et al., 2004; Zheng et al., 2009). Other vertebrates, including mammals, do not have the capability to synthesize PUFAs and highly unsaturated fatty acids (HUFAs), so they need to absorb them from diet (Castro et al., 2012). Since fish are the major source of PUFAs and HUFAs for humans (González-Rovira et al., 2009), it is important to know how these fatty acids could affect peptides involved in food intake and metabolism regulation, to use all these information to improve aquaculture yield. In mammals, oleic acid inhibits the *NUCB2/nesfatin-1* mRNA expression whereas increases the *ghrelin* mRNA expression *in vitro* (Mohan et al., 2014). Also, the long-chain fatty acid sensing receptor GPR120 co-localizes with ghrelin containing cells in the mice duodenum and

has been shown to play a role in the lipid-sensing cascade of these cells. Free fatty acid receptor 1 (FFAR1), involved in sensing of long/medium chain fatty acids, is expressed only in cells containing the des-acyl (therefore, non-active) ghrelin in the stomach of mice, and its function is unclear (Janssen et al., 2012). We found that *preproghrelin* mRNA expression in the intestine is mainly upregulated by oleic acid, but an increase in the chain length and saturation level of fatty acids led to a decrease in its expression, especially after 2 h of treatment. The role of all fatty acids on NUCB2/nesfatin-1, regardless of their chain length and unsaturation level, is primarily inhibitory, except for oleic acid. Overall, results from this study indicate that in goldfish intestine, fatty acids are important regulators of appetite regulatory peptides.

In the hepatopancreas, we observed a clear indication of a concentration- and time-dependent control of ghrelin and NUCB2/nesfatin-1 by fatty acids. For instance, 2 h of treatment with 10 μM oleic acid upregulated preproghrelin mRNA and protein expression, but 1 μM and 100 μM oleic acid caused the opposite effect. EPA downregulated *preproghrelin* expression at 2 h, but led to an upregulation at 4 h. This type of interaction indicates that both peptides could be acting together in the regulation of some metabolic processes that depend on the time of exposure to fatty acids. Although more experiments are needed in order to determine the role of ghrelin and nesfatin-1 in hepatopancreas, present results indicate that fatty acids clearly modulate their mRNA and protein expression in a concentration- and time-dependent manner. Moreover, the mRNA expression assay indicates a global decrease in the expression of *preproghrelin* and an increase in the expression of *NUCB2/nesfatin-1* in response to fatty acids. Data from our group showed that ghrelin co-localizes with lipoprotein lipase, essential for degrading dietary lipids, and modifies its mRNA and protein expression in goldfish hepatopancreas (Blanco et al., 2017). Taken this into consideration, it is likely that ghrelin and nesfatin-1 play a role in lipid metabolism.

Our results in general show a decrease in intestinal ghrelin mRNA expression after 2 h of treatment with different macronutrient components. This result is in agreement with the decrease in plasma ghrelin observed after meals (Bodosi et al., 2004; Cummings et al., 2001; LeSauter et al., 2009). Since nesfatin-1 is an anorexigenic peptide, an increase after treatment with macronutrients was anticipated. The decrease in NUCB2/nesfatin-1 mRNA and protein expression observed after 2 h in the intestine could indicate another function of this peptide not related to food intake control. *In vivo* and *in vitro* studies demonstrated that macronutrients regulate genes involved in growth and metabolism, particularly in the hepatopancreas (Alvarez et al., 2000; Douros et al., 2016; Reinecke, 2010; Riley et al., 2009a, b). In this tissue, the ghrelin and NUCB2/nesfatin-1 expression seems to be coordinated, showing a decrease in ghrelin mRNA expression and an increase in NUCB2/nesfatin-1 mRNA and protein expression after 2 and 4 h of treatment with different macronutrients. More studies are required to elucidate the mechanisms of macronutrient regulation of ghrelin and nesfatin-1, but it could be hypothesized that macronutrients in general exerts an anorexigenic effect in the intestine which is the main producer of peripheral ghrelin, while the coordinated expression of both peptides found in the hepatopancreas suggest an involvement in other process such as metabolism regulation. Overall, the knowledge created here will help develop endocrine-based considerations, especially in terms of designing fish diets aiming to improve fish growth.

Funding

This work was supported by a Discovery Grant, and Discovery Accelerator Supplement award from the Natural Sciences and Engineering Research Council of Canada (NSERC), an Establishment grant from Saskatchewan Health Research Foundation (SHRF) and John R. Evans Leaders Fund from the Canada Foundation for Innovation to S. Unniappan. S. Unniappan is a recipient of the CIHR New Investigator Award. J.I. Bertucci is a graduate fellow supported by the Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Argentina. A.M. Blanco is a graduate fellow funded by the Spanish Ministerio de Educación y Ciencia.

Conflict of interest

The authors declare that they have no conflict of interest.

Uncited references

Enes et al., 2009
Palmer and Ryman, 1972
Polakof et al., 2012
Shlimun and Unniappan, 2011

References

Abi-Ayad, A., Kestemont, P., 1994. Comparison of the nutritional status of goldfish (Carassius auratus) larvae fed with live, mixed or dry diet. Aquaculture 128, 163–176. http://dx.doi.org/10.1016/0044-8486(94)90111-2.

Akiyama, T., Oohara, I., Yamamoto, T., 1997. Comparison of essential amino acid requirements with A/E ratio among fish species (review paper). Fish. Sci. 63, 963–970. http://dx.doi.org/10.2331/fishsci.63.963.

Al Massadi, O., Lear, P.V., Müller, T.D., López, M., Diéguez, C., Tschöp, M.H., Nogueiras, R., 2014. Review of novel aspects of the regulation of ghrelin secretion. Curr. Drug Metab.

Alvarez, M.J., Diez, a., Lopez Bote, C., Gallego, M., Bautista, J.M., 2000. Short-term modulation of lipogenesis by macronutrients in rainbow trout (Oncorhynchus mykiss) hepatocytes. Br. J. Nutr. 84, 619–628 (doi:S0007114500001951 [pii).

Asakawa, A., Inui, A., Kaga, T., Yuzuriha, H., Nagata, T., Ueno, N., Makino, S., Fujimiya, M., Niijima, A., Fujino, M.A., Kasuga, M., 2001. Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin. Gastroenterology 120, 337–345. http://dx.doi.org/10.1053/gast.2001.22158.

Asakawa, A., Inui, A., Fujimiya, M., Sakamaki, R., Shinfuku, N., Ueta, Y., Meguid, M.M., Kasuga, M., 2005. Stomach regulates energy balance via acylated ghrelin and desacyl ghrelin. Gut 54, 18–24. http://dx.doi.org/10.1136/gut.2004.038737.

Beck, B., Richy, S., 2008. Differential long-term dietary regulation of adipokines, ghrelin, or corticosterone: impact on adiposity. J. Endocrinol. 196, 171–179. http://dx.doi.org/10.1677/JOE-07-0468.

Beck, B., Musse, N., Stricker-Krongrad, A., 2002. Ghrelin, macronutrient intake and dietary preferences in long-evans rats. Biochem. Biophys. Res. Commun. 292, 1031–1035. http://dx.doi.org/10.1006/bbrc.2002.6737.

Blanco, A.M., Bertucci, J.I., Delgado, M.J., Valenciano, A.I., Unniappan, S., 2016. . Comp. Biochem. Physiol. A 195, 1–9. http://dx.doi.org/10.1016/j.cbpa.2016.01.016.

Blanco, A.M., Bertucci, J.I., Delgado, M.J., Valenciano, A.I., Unniappan, S., 2016. Tissue-specific expression of ghrelinergic and NUCB2/nesfatin-1 systems in goldfish (Carassius auratus) is modulated by macronutrient composition of diets. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 195, 1–9. http://dx.doi.org/10.1016/j.cbpa.2016.01.016.

Blom, W.A.M., Stafleu, A., De Graaf, C., Kok, F.J., Schaafsma, G., Hendriks, H.F.J., 2005. Ghrelin response to carbohydrate-enriched breakfast is related to insulin. Am. J. Clin. Nutr. 81, 367–375.

Bodosi, B., Gardi, J., Hajdu, I., Szentirmai, E., Obal, F., Krueger, J.M., 2004. Rhythms of ghrelin, leptin, and sleep in rats: effects of the normal diurnal cycle, restricted feeding, and sleep deprivation. Am. J. Physiol. Regul. Integr. Comp. Physiol. 287, R1071–R1079. http://dx.doi.org/10.1152/ajpregu.00294.2004.

Borlongan, I.G., Coloso, R.M., 1993. Requirements of juvenile milkfish (Chanos chanos Forsskal) for essential amino acids. J. Nutr. 123, 125–132.

Castro, L.F.C., Monroig, , Leaver, M.J., Wilson, J., Cunha, I., Tocher, D.R., 2012. Functional desaturase Fads1 (Δ5) and Fads2 (Δ6) orthologues evolved before the

origin of jawed vertebrates. PLoS One 7, e31950http://dx.doi.org/10.1371/journal.pone.0031950.
Cowey, C.B., 1992. Nutrition: estimating requirements of rainbow trout. Aquaculture 100, 177–189. http://dx.doi.org/10.1016/0044-8486(92)90370-Z.
Cummings, D.E., Purnell, J.Q., Frayo, R.S., Schmidova, K., Wisse, B.E., Weigle, D.S., 2001. A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. Diabetes 50, 1714–1719. http://dx.doi.org/10.2337/diabetes.50.8.1714.
Dezaki, K., 2013. Ghrelin function in insulin release and glucose metabolism. Endocr. Dev. 25, 135–143. http://dx.doi.org/10.1159/000346064.
di Rienzo, J.A., Casanoves, F., Balzarini, M., Gonzalez, L., Tablada, M., Robledo, C., 2013. Infostat - Sofware estadístico. Universidad Nacional de Córdoba, Argentina [WWW Document]. Univ. Nac. Córdoba, Argentina (URL http://www.infostat.com.ar/).
Diotel, N., Servili, A., Gueguen, M.M., Mironov, S., Pellegrini, E., Vaillant, C., Zhu, Y., Kah, O., Anglade, I., 2011. Nuclear progesterone receptors are up-regulated by estrogens in neurons and radial glial progenitors in the brain of zebrafish. PLoS One 6. http://dx.doi.org/10.1371/journal.pone.0028375.
Douros, J.D., Baltzegar, D.A., Mankiewicz, J., Taylor, J., Yamaguchi, Y., Lerner, D.T., Seale, A.P., Grau, E.G., Breves, J.P., Borski, R.J., 2016. Control of leptin by metabolic state and its regulatory interactions with pituitary growth hormone and hepatic growth hormone receptors and insulin like growth factors in the tilapia (Oreochromis mossambicus). Gen. Comp. Endocrinol. http://dx.doi.org/10.1016/j.ygcen.2016.07.017.
.
García-Galiano, D., Navarro, V.M., Gaytan, F., Tena-Sempere, M., 2010. Expanding roles of NUCB2/nesfatin-1 in neuroendocrine regulation. J. Mol. Endocrinol. 45, 281–290. http://dx.doi.org/10.1677/JME-10-0059.
García-Galiano, D., Navarro, V.M., Roa, J., Ruiz-Pino, F., Sánchez-Garrido, M.A., Pineda, R., Castellano, J.M., Romero, M., Aguilar, E., Gaytán, F., Diéguez, C., Pinilla, L., Tena-Sempere, M., 2010. The anorexigenic neuropeptide, nesfatin-1, is indispensable for normal puberty onset in the female rat. J. Neurosci. 30, 7783–7792. http://dx.doi.org/10.1523/JNEUROSCI.5828-09.2010.
Goebel, M., Stengel, A., Wang, L., Taché, Y., 2011. Central nesfatin-1 reduces the nocturnal food intake in mice by reducing meal size and increasing inter-meal intervals. Peptides 32, 36–43. http://dx.doi.org/10.1016/j.peptides.2010.09.027.
Gomez, G., Han, S., Englander, E.W., Greeley, G.H., 2012. Influence of a long-term high-fat diet on ghrelin secretion and ghrelin-induced food intake in rats. Regul. Pept. 173, 60–63.
Gonzalez, R., Kerbel, B., Chun, A., Unniappan, S., 2010. Molecular, cellular and physiological evidences for the anorexigenic actions of nesfatin-1 in goldfish. PLoS One 5, e15201http://dx.doi.org/10.1371/journal.pone.0015201.
Gonzalez, R., Perry, R.L.S., Gao, X., Gaidhu, M.P., Tsushima, R.G., Ceddia, R.B., Unniappan, S., 2011. Nutrient responsive nesfatin-1 regulates energy balance and induces glucose-stimulated insulin secretion in rats. Endocrinology 152, 3628–3637. http://dx.doi.org/10.1210/en.2010-1471.
Gonzalez, R., Mohan, H., Unniappan, S., 2012. Nucleobindins: bioactive precursor proteins encoding putative endocrine factors?. Gen. Comp. Endocrinol. 176, 341–346. http://dx.doi.org/10.1016/j.ygcen.2011.11.021.
González-Rovira, A., Mourente, G., Zheng, X., Tocher, D.R., Pendón, C., 2009. Molecular and functional characterization and expression analysis of a Δ6 fatty acyl desaturase cDNA of European Sea Bass (Dicentrarchus labrax L.). Aquaculture 298, 90–100. http://dx.doi.org/10.1016/j.aquaculture.2009.10.012.
Green, J.A., Hardy, R.W., 2002. The optimum dietary essential amino acid pattern for rainbow trout (Oncorhynchus mykiss), to maximize nitrogen retention and minimize nitrogen excretion. Fish Physiol. Biochem. 27, 97–108. http://dx.doi.org/10.1023/B:FISH.0000021878.81647.6e.
Handjieva-Darlenska, T., Boyadjieva, N., 2009. The effect of high-fat diet on plasma ghrelin and leptin levels in rats. J. Physiol. Biochem. 65, 157–164. http://dx.doi.org/10.1007/BF03179066.
Hemre, G.I., Mommsen, T.P., Krogdahl, , 2002. Carbohydrates in fish nutrition: effects on growth, glucose metabolism and hepatic enzymes. Aquac. Nutr. 8, 175–194. http://dx.doi.org/10.1046/j.1365-2095.2002.00200.x.
Horvath, T.L., Diano, S., Sotonyi, P., Heiman, M., Tschöp, M., 2001. Minireview: ghrelin and the regulation of energy balance—a hypothalamic perspective. Endocrinology 142, 4163–4169.
Janssen, S., Laermans, J., Iwakura, H., Tack, J., Depoortere, I., 2012. Sensing of fatty acids for octanoylation of ghrelin involves a gustatory G-protein. PLoS One 7, http://dx.doi.org/10.1371/journal.pone.0040168.
Jönsson, E., 2013. The role of ghrelin in energy balance regulation in fish. Gen. Comp. Endocrinol. 187, 79–85. http://dx.doi.org/10.1016/j.ygcen.2013.03.013.
Jönsson, E., Forsman, A., Einarsdottir, I.E., Kaiya, H., Ruohonen, K., Björnsson, B.T., 2007. Plasma ghrelin levels in rainbow trout in response to fasting, feeding and food composition, and effects of ghrelin on voluntary food intake. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 147, 1116–1124. http://dx.doi.org/10.1016/j.cbpa.2007.03.024.
Kaiya, H., Miyazato, M., Kangawa, K., Peter, R.E., Unniappan, S., 2008. Ghrelin: a multifunctional hormone in non-mammalian vertebrates. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 149, 109–128. http://dx.doi.org/10.1016/j.cbpa.2007.12.004.
Kang, K.S., Yahashi, S., Matsuda, K., 2011. Central and peripheral effects of ghrelin on energy balance, food intake and lipid metabolism in teleost fish. Peptides http://dx.doi.org/10.1016/j.peptides.2011.05.006.
Kerbel, B., Unniappan, S., 2012. Nesfatin-1 suppresses energy intake, Co-localises ghrelin in the brain and gut, and alters ghrelin, cholecystokinin and orexin mRNA expression in goldfish. J. Neuroendocrinol. 24, 366–377. http://dx.doi.org/10.1111/j.1365-2826.2011.02246.x.
Kinzig, K.P., Hargrave, S.L., Hyun, J., Moran, T.H., 2007. Energy balance and hypothalamic effects of a high-protein/low-carbohydrate diet. Physiol. Behav. 92, 454–460. http://dx.doi.org/10.1016/j.physbeh.2007.04.019.
Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., Kangawa, K., 1999. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402, 656–660. http://dx.doi.org/10.1038/45230.
Lauff, M., Hofer, R., 1984. Proteolytic enzymes in fish development and the importance of dietary enzymes. Aquaculture 37, 335–346. http://dx.doi.org/10.1016/0044-8486(84)90298-9.
Lejeune, M.P.G.M., Westerterp, K.R., Adam, T.C.M., Luscombe-Marsh, N.D., Westerterp-Plantenga, M.S., 2006. Ghrelin and glucagon-like peptide 1 concentrations, 24-h satiety, and energy and substrate metabolism during a high-protein diet and measured in a respiration chamber. Am. J. Clin. Nutr. 83, 89–94. http://dx.doi.org/10.1152/ajpgi.00478.2011.
LeSauter, J., Hoque, N., Weintraub, M., Pfaff, D.W., Silver, R., 2009. Stomach ghrelin-secreting cells as food-entrainable circadian clocks. Proc. Natl. Acad. Sci. U. S. A. 106, 13582–13587. http://dx.doi.org/10.1073/pnas.0906426106.
Li, Z., Gao, L., Tang, H., Yin, Y., Xiang, X., Li, Y., Zhao, J., Mulholland, M., Zhang, W., 2013. Peripheral effects of nesfatin-1 on glucose homeostasis. PLoS One 8, http://dx.doi.org/10.1371/journal.pone.0071513.
Lin, F., Zhou, C., Chen, H., Wu, H., Xin, Z., Liu, J., Gao, Y., Yuan, D., Wang, T., Wei, R., Chen, D., Yang, S., Wang, Y., Pu, Y., Li, Z., 2014. Molecular characterization, tissue distribution and feeding related changes of NUCB2A/nesfatin-1 in Ya-fish (Schizothorax prenanti). Gene 536, 238–246. http://dx.doi.org/10.1016/j.gene.2013.12.031.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and. Methods 25, 402–408. http://dx.doi.org/10.1006/meth.2001.1262.
Lochmann, R.T., Phillips, H., 1994. Dietary protein requirement of juvenile golden shiners (Notemigonus crysoleucas) and goldfish (Carassius auratus) in aquaria. Aquaculture 128, 277–285. http://dx.doi.org/10.1016/0044-8486(94)90317-4.
Matsuda, K., Miura, T., Kaiya, H., Maruyama, K., Shimakura, S., Uchiyama, M., Kangawa, K., Shioda, S., 2006. Regulation of food intake by acyl and des-acyl ghrelins in the goldfish. Peptides 27, 2321–2325. http://dx.doi.org/10.1016/j.peptides.2006.03.028.
Mohan, H., Unniappan, S., 2012. Ontogenic pattern of nucleobindin-2/nesfatin-1 expression in the gastroenteropancreatic tissues and serum of Sprague Dawley rats. Regul. Pept. 175, 61–69. http://dx.doi.org/10.1016/j.regpep.2012.02.006.
Mohan, H., Unniappan, S., 2013. Phylogenetic aspects of nucleobindin-2/nesfatin-1. Curr. Pharm. Des. 19, 6929–6934.
Mohan, H., Ramesh, N., Mortazavi, S., Le, A., Iwakura, H., Unniappan, S., 2014. Nutrients differentially regulate Nucleobindin-2/nesfatin-1 in vitro in cultured stomach ghrelinoma (MGN3-1) cells and in vivo in male mice. PLoS One 9, e115102http://dx.doi.org/10.1371/journal.pone.0115102.
Moon, T.W., 2001. Glucose intolerance in teleost fish: fact or fiction?. Comp. Biochem. Physiol. B. Biochem. Mol. Biol. 129, 243–249. http://dx.doi.org/10.1016/S1096-4959(01)00316-5.
Mortazavi, S., Gonzalez, R., Ceddia, R., Unniappan, S., 2015. Long-term infusion of nesfatin-1 causes a sustained regulation of whole-body energy homeostasis of male Fischer 344 rats. Front. Cell Dev. Biol. 3, 1–12. http://dx.doi.org/10.3389/fcell.2015.00022.
Nakagawa, E., Nagaya, N., Okumura, H., 2002. Hyperglycaemia suppresses the secretion of ghrelin, a novel growth-hormone-releasing peptide: responses to the intravenous and oral administration of glucose. Clin. Sci. 3, 325–328. http://dx.doi.org/10.1042/cs1030325.
Nieuwenhuizen, A.G., Hochstenbach-Waelen, A., Veldhorst, M.a.B., Westerterp, K.R., Engelen, M.P.K.J., Brummer, R.-J.M., Deutz, N.E.P., Westerterp-Plantenga, M.S., 2009. Acute effects of breakfasts containing alpha-lactalbumin, or gelatin with or without added tryptophan, on hunger, “satiety” hormones and amino acid profiles. Br. J. Nutr. 101, 1859–1866. http://dx.doi.org/10.1017/S0007114508131774.
Nunes, A.J.P., Sá, M.V.C., Browdy, C.L., Vazquez-Anon, M., 2014. Practical supplementation of shrimp and fish feeds with crystalline amino acids. Aquaculture http://dx.doi.org/10.1016/j.aquaculture.2014.04.003.
Oh-I, S., Shimizu, H., Satoh, T., Okada, S., Adachi, S., Inoue, K., Eguchi, H., Yamamoto, M., Imaki, T., Hashimoto, K., Tsuchiya, T., Monden, T., Horiguchi, K., Yamada, M., Mori, M., 2006. Identification of nesfatin-1 as a satiety molecule in the hypothalamus. Nature 443, 709–712.
.

Perez-sanchez, J., Martipalanca, H., Kaushik, S.J., 1995. Ration size and protein-intake affect circulating growth-hormone concentration, hepatic growth-hormone binding and plasma insulin-like growth-factor-I immunoreactivity in a marine teleost, the gilthead sea bream (Sparus-Aurata). J. Nutr. 125, 546–552.

.

Ramesh, N., Mortazavi, S., Unniappan, S., 2015. Nesfatin-1 stimulates glucagon-like peptide-1 and glucose-dependent insulinotropic polypeptide secretion from STC-1 cells in vitro. Biochem. Biophys. Res. Commun. 462, 124–130. http://dx.doi.org/10.1016/j.bbrc.2015.04.100.

Reinecke, M., 2010. Influences of the environment on the endocrine and paracrine fish growth hormone-insulin-like growth factor-I system. J. Fish Biol. 76, 1233–1254. http://dx.doi.org/10.1111/j.1095-8649.2010.02605.x.

.

Riley, L.G., Walker, A.P., Dorough, C.P., Schwandt, S.E., Grau, E.G., 2009. Glucose regulates ghrelin, neuropeptide Y, and the GH/IGF-I axis in the tilapia, Oreochromis mossambicus. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 154, 541–546. http://dx.doi.org/10.1016/j.cbpa.2009.08.018.

Roy, S.S., Mukherjee, M., Bhattacharya, S., Mandal, C.N., Kumar, L.R., Dasgupta, S., Bandyopadhyay, I., Wakabayashi, K., 2003. A new cell secreting insulin. Endocrinology 144, 1585–1593. http://dx.doi.org/10.1210/en.2002-220350.

Sánchez-Bretaño, A., Alonso-Gómez, Á.L., Delgado, M.J., Isorna, E., 2015. The liver of goldfish as a component of the circadian system: integrating a network of signals. Gen. Comp. Endocrinol.http://dx.doi.org/10.1016/j.ygcen.2015.05.001.

Sánchez-Bretaño, A., Blanco, A.M., Unniappan, S., Kah, O., Gueguen, M.-M., Bertucci, J.I., Alonso-Gómez, Á.L., Valenciano, A.I., Isorna, E., Delgado, M.J., 2015. In situ localization and rhythmic expression of ghrelin and ghs-r1 ghrelin receptor in the brain and gastrointestinal tract of goldfish (Carassius auratus). PLoS One 10, e0141043http://dx.doi.org/10.1371/journal.pone.0141043.

Sánchez-Bretaño, A., Callejo, M., Montero, M., Alonso-Gómez, Á.L., Delgado, M.J., Isorna, E., 2016. Performing a hepatic timing signal: glucocorticoids induce gper1a and gper1b expression and repress gclock1a and gbmal1a in the liver of goldfish. J. Comp. Physiol. B. 186, 73–82. http://dx.doi.org/10.1007/s00360-015-0936-2.

Shiiya, T., Nakazato, M., Mizuta, M., Date, Y., Mondal, M.S., Tanaka, M., Nozoe, S.-I., Hosoda, H., Kangawa, K., Matsukura, S., 2002. Plasma ghrelin levels in lean and obese humans and the effect of glucose on ghrelin secretion. J. Clin. Endocrinol. Metab. 87, 240–244. http://dx.doi.org/10.1210/jcem.87.1.8129.

Shimizu, H., Oh-I, S., Hashimoto, K., Nakata, M., Yamamoto, S., Yoshida, N., Eguchi, H., Kato, I., Inoue, K., Satoh, T., Okada, S., Yamada, M., Yada, T., Mori, M., 2009. Peripheral administration of nesfatin-1 reduces food intake in mice: the leptin-independent mechanism. Endocrinology 150, 662–671. http://dx.doi.org/10.1210/en.2008-0598.

Shlimun, A., Unniappan, S., 2011. Ghrelin o-acyl transferase: bridging ghrelin and energy homeostasis. Int. J. Pept. 2011, 217957. http://dx.doi.org/10.1155/2011/217957.

Stengel, A., Goebel, M., Wang, L., Taché, Y., 2010. Ghrelin, des-acyl ghrelin and nesfatin-1 in gastric X/A-like cells: role as regulators of food intake and body weight. Peptides 31, 357–369. http://dx.doi.org/10.1016/j.peptides.2009.11.019.

Sugino, T., Hasegawa, Y., Kurose, Y., Kojima, M., Kangawa, K., Terashima, Y., 2004. Effects of ghrelin on food intake and neuroendocrine function in sheep. Anim. Reprod. Sci. 82–83, 183–194. http://dx.doi.org/10.1016/j.anireprosci.2004.05.001.

Tang-Christensen, M., Vrang, N., Ortmann, S., Bidlingmaier, M., Horvath, T.L., Tschöp, M., 2004. Central administration of ghrelin and agouti-related protein (83–132) increases food intake and decreases spontaneous locomotor activity in rats. Endocrinology 145, 4645–4652. http://dx.doi.org/10.1210/en.2004-0529.

Tibaldi, E., Kaushik, S.J., 2005. Amino acid requirements of Mediterranean fish species. Cah. Options Méditérr. 63, 59–65.

Tschöp, M., Smiley, D.L., Heiman, M.L., 2000. Ghrelin induces adiposity in rodents. Nature 407, 908–913. http://dx.doi.org/10.1038/35038090.

Unniappan, S., Canosa, L.F., Peter, R.E., 2004. Orexigenic actions of ghrelin in goldfish: feeding-induced changes in brain and gut mRNA expression and serum levels, and responses to central and peripheral injections. Neuroendocrinology 79, 100–108. http://dx.doi.org/10.1159/000076634.

Vagner, M., Santigosa, E., 2011. Characterization and modulation of gene expression and enzymatic activity of delta-6 desaturase in teleosts: a review. Aquaculture 315, 131–143. http://dx.doi.org/10.1016/j.aquaculture.2010.11.031.

Velasco, C., Librán-Pérez, M., Otero-Rodiño, C., López-Patiño, M.A., Míguez, J.M., Cerdá-Reverter, J.M., Soengas, J.L., 2016. Ghrelin modulates hypothalamic fatty acid-sensing and control of food intake in rainbow trout. J. Endocrinol. 228, 25–37. http://dx.doi.org/10.1530/JOE-15-0391.

Veldhorst, M., Smeets, A., Soenen, S., Hochstenbach-Waelen, A., Hursel, R., Diepvens, K., Lejeune, M., Luscombe-Marsh, N., Westerterp-Plantenga, M., 2008. Protein-induced satiety: effects and mechanisms of different proteins. Physiol. Behav. http://dx.doi.org/10.1016/j.physbeh.2008.01.003.

Wang, X., Ni, S., Xu, Y., Liang, L., Du, L., Gu, W., 2012. Effects of long-term high-fat/high-energy and high-protein diets on insulin and ghrelin expression in developing rats. Endocr. Res. 37, 97–109.

Wren, A.M., Small, C.J., Ward, H.L., Murphy, K.G., Dakin, C.L., Taheri, S., Kennedy, A.R., Roberts, G.H., Morgan, D.G.A., Ghatei, M.A., Bloom, S.R., 2000. The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. Endocrinology 141, 4325–4328. http://dx.doi.org/10.1210/en.141.11.4325.

Zheng, X., Seiliez, I., Hastings, N., Tocher, D.R., Panserat, S., Dickson, C.A., P.B., A.J.T., 2004. Characterisation and Comparison of Fatty Acyl Δ6 Desaturase cDNAs From Freshwater and Marine Teleost Fish Species. 139, 269–279. http://dx.doi.org/10.1016/j.cbpc.2004.08.003.Characterisation.

Zheng, X., Ding, Z., Xu, Y., Monroig, O., Morais, S., Tocher, D.R., 2009. Physiological roles of fatty acyl desaturases and elongases in marine fish: characterisation of cDNAs of fatty acyl Δ6 desaturase and elovl5 elongase of cobia (Rachycentron canadum). Aquaculture 290, 122–131. http://dx.doi.org/10.1016/j.aquaculture.2009.02.010.